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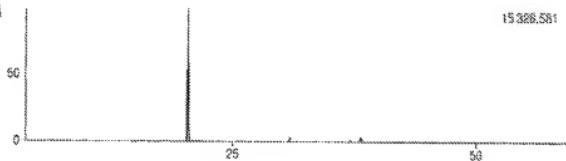
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(54) Polyhydroxyalkanoate-containing structure and manufacturing method thereof

(57) A method for manufacturing polyhydroxyalkanoate-containing structure, at least a part of a base material surface of the structure being coated with polyhydroxyalkanoate, the method comprises the steps of immobilizing a polyhydroxyalkanoate synthase on the base material surface, synthesizing, on the base material surface, polyhydroxyalkanoate using a 3-hydroxyacyl coenzyme A to become the substrate of the synthase and the synthase and coating at least a part of the base material surface with the synthesized polyhydroxy-

kananoate, wherein the synthase contains an amino acid sequence capable of binding to the base material. A polyhydroxyalkanoate-containing structure, at least a part of a base material surface of the structure being coated with a polyhydroxyalkanoate, comprises the base material, a polyhydroxyalkanoate synthase immobilized on the base material surface, and the polyhydroxyalkanoate with which at least a part of the base material surface is coated, wherein the synthase contains an amino acid sequence capable of binding to the base material.

FIG. 1A



15326,581

[0002]

Description**BACKGROUND OF THE INVENTION**5 **Field of the invention**

[0001] The present invention relates to a method for manufacturing polyhydroxyalkanoate-containing structure comprising the steps of immobilizing on a base material polyhydroxyalkanoate synthase involved in polyhydroxyalkanoate biosynthesis reaction and coating at least part of the base material with polyhydroxyalkanoate by polymerizing a 3-hydroxyacyl coenzyme A by use of the enzyme to synthesize the polyhydroxyalkanoate. More particularly, the invention relates to a method for manufacturing polyhydroxyalkanoate-containing structure by immobilizing to a base material polyhydroxyalkanoate synthase containing an amino acid sequence capable of binding to the base material.

[0002] The present invention relates to a structure that has polyhydroxyalkanoate, base material and polyhydroxy-alkanoate synthase immobilized on the base material, the polyhydroxyalkanoate coating at least part of the base material. The structure of the present invention encompasses a granular structure (hereinafter called a "capsular structure") in which polyhydroxyalkanoate is coated on a granular base material, and a plate- or film-like structure (hereinafter called a "terminated structure") in which at least part of a plate- or film-like base material is coated with polyhydroxyalkanoate.

[0003] The structure of the present invention can find a wide range of applications as a functional structure. For example, the capsular structure can have a large number of applications as a variety of functional structures such as a pigment dispersant of excellent dispersion stability and a toner for electrophotography of excellent electrostatic property, and the laminated structure as various functional structures including an OHP film and an electronic device.

Related Background Art

25 [0004] Polymeric materials are essential to modern industries and our lives. The materials, which are inexpensive and lightweight and have good moldability, are widely utilized as packaging material and cushioning material, or fiber material, as well as boxes for household electrical appliances. On the other hand, diverse functional materials such as a liquid crystal material and a coal agent are also obtained by utilizing stable properties of these polymeric materials to thereby place substitutions or exhibiting various functions on molecular chains of the polymers. These functional materials are higher in added values than polymers for structural materials and thus can be expected to have large market needs even in a small amount. These functional polymeric materials have been produced so far by organic, synthetic chemical methods in synthetic processes of polymers or by modifying synthesized polymers with substituents. Polymers of basic frameworks for functional polymeric materials have been obtained from petroleum based raw material by organic, synthetic chemical methods in most cases. Typical examples of these polymers include polyethylene, poly(ethylene terephthalate), polyesters, polystyrene, poly(vinyl chloride) and polyacrylamides.

[0005] Incidentally, the present inventors have focused on a multilayered structure, the base material of the structures being coated with a polymeric compound, as a basic element that imparts large added values to the polymeric compound. A composite structure of extremely useful functionality can be obtained by coating a specific base material with a polymeric compound.

[0006] While polymeric compounds used for coating base materials are conventionally synthesized and made to be structures by organic synthetic processes, and then various functions are added to them, recently, the production of polymeric compounds by biengineering approaches has been actively studied and part of it is operational. Known examples include as polymeric compounds derived from microbes polyhydroxyalkanoates (hereinafter sometimes abbreviated as PHAs) such as poly-3-hydroxy-n-butyric acid (hereinafter sometimes abbreviated as PHB), and a copolymer of 3-hydroxy-n-butyric acid and 3-hydroxy-n-valeric acid (hereinafter sometimes abbreviated as PHBV), polysaccharides such as bacterial cellulose and pullulan, and polyamino acids such as poly- γ -glutamic acid and polylysine. In particular, PHAs can be utilized for various products by melt processing, or the like, like conventional plastics and also exhibit excellent biocompatibility, thus being expected to find applications including flexible materials for medical treatment.

[0007] Recently, an attempt has been started to synthesize PHAs *in vitro* by taking the aforementioned PHB synthase or PHA synthase out of the microbe.

[0008] For example, a PHB composed of a 3-hydroxy-n-butyric acid unit has been successfully synthesized by causing the action of 3-hydroxybutyryl CoA on a PHB synthase derived from *Alcaligenes eutrophus* (Proc. Natl. Acad. Sci. USA, 92, 8279-8283, 1995). In addition, PHBs composed of a 3-hydroxy-n-butyric acid unit or a 3-hydroxy-n-valeric acid unit has been successfully synthesized by causing the action of 3-hydroxybutyryl CoA or 3-hydroxyvaleryl CoA on a PHB synthase derived from *Alcaligenes eutrophus* (Int. J. Biol. Macromol., 25, 55-60, 1999). Furthermore, in this study, a PHG composed only of the R form of a 3-hydroxy-n-butyric acid unit was synthesized, due to stereo-selectivity.

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CLP/M
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BRIEF DESCRIPTION OF THE DRAWINGS

[0017]

- 5 Figs. 1A and 1B show GC-MS analysis results of the outer shell of a PHA capsular structure using copper phthalocyanine in Example 4.
 Figs. 2A and 2B show GC-MS analysis results of the outer shell of a PHA capsular structure using carbon black in Example 8.
 10 Figs. 3A and 3B show GC-MS analysis results of the laminated body of a PHA laminated structure using a silicon board in Example 12.
 Fig. 4 shows a schematic diagram of a image forming apparatus having a reuse mechanism of a toner.
 Fig. 5 shows a sectional view of the main portion of a developing apparatus for a one-component developing agent;
 Fig. 6 shows an exploded perspective view of the main portion of a fixation apparatus;
 15 Fig. 7 shows an enlarged sectional view of the main portion that illustrates a film condition when the fixation apparatus is not run, and

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

- [0018] In the present invention, for a base material to be coated with a PHA, if it can immobilize a PHA synthase, a general polymeric compound or inorganic solid material, e.g., resin, glass or a metal, can be selected, as required, and used. The kind or structure of a base material can be selected, as required, and used according to a method of immobilizing a PHA synthase, the form of application of a produced structure, etc.
- [0019] Examples of the granular base material (core) include resin particulates produced by polymerizing polymerizable monomers selected from the group consisting of styrene base polymerizable monomers such as styrene, α -methylstyrene, β -methylstyrene, α -methylstyrene, α -methylstyrene, β -methylstyrene, 2,4-dimethylstyrene, α -n-butylstyrene, α -tert-butylstyrene, α -n-hexylstyrene, α -n-octylstyrene, α -n-nonylstyrene, α -n-decylstyrene, α -n-dodecylstyrene, α -methoxystyrene, and α -phenylstyrene, acrylic polymerizable monomers such as methyl acrylate, ethyl acrylate, n -propyl acrylate, iso-propyl acrylate, n -butyl acrylate, t -butyl acrylate, n -amyl acrylate, n -hexyl acrylate, 2-ethylhexyl acrylate, n -octyl acrylate, n -nonyl acrylate, cyclohexyl acrylate, benzyl acrylate, dimethylphosphate ethyl acrylate, diethylphosphate ethyl acrylate, diethylphosphate ethyl acrylate, and 2-benzoyloxyethyl acrylate, methacrylic polymerizable monomers such as methyl methacrylate, ethyl methacrylate, n -propyl methacrylate, iso-propyl methacrylate, n -butyl methacrylate, iso-butyl methacrylate, t -butyl methacrylate, n -amyl methacrylate, n -hexyl methacrylate, 2-ethylhexyl methacrylate, n -octyl methacrylate, n -nonyl methacrylate, diethylphosphate ethyl methacrylate, and dibutylphosphate ethyl methacrylate, vinyl base polymerizable monomers including methylene aliphatic monocarboxylates, vinyl ethers such as vinyl acetate, vinyl propionate, vinyl benzoate, vinyl butyrate, vinyl benzoate, and vinyl formate, vinyl ethers such as vinylmethyl ether, vinylethyl ether, and vinylisobutyl ether, vinyl ketones such as vinyl methyl ketone, vinyl hexyl ketone, and vinyl isopropyl ketone; resin particulates produced by adding to the above described monomers a variety of additives such as polymers of polar groups and esterants; particulates including paraffin wax, polyolefin wax, Fischer Tropischen wax, amide wax, higher fatty acids, ester wax, derivatives thereof, graft compounds thereof, and block compounds thereof; clay minerals such as kaolinite, bentonite, talc, and micor; metal oxides such as alumina and titanium dioxide; inclusions inorganic salts such as silica gel, hydroxyapatite, and calcium phosphate gel; black pigments such as carbon black, copper oxide, manganese dioxide, anime black, activated carbon, nonmagnetic ferrite, and magnetite; yellow pigments such as Chrome Yellow, Zinc Yellow, Iron Oxide Yellow, Cadmium Yellow, Mineral Fast Yellow, Nickel Titanium Yellow, Nectur Yellow, Naphtol Yellow S, Hansa Yellow G, Hansa Yellow 10G, Benzidine Yellow G, Benzidine Yellow GR, Quinoline Yellow Lake, Permanent Yellow NCG, and Turtledove Lake, orange pigments such as Orange Chrome, Molybdenum Orange, Permanent Orange GTR, Pyrazolone Orange, Vulcan Orange, Benjamine Orange G, Indanthrene Brilliant Orange HK, and Indanthrene Brilliant Orange GK, red pigments such as Red Iron Oxide, Cadmium Red Lead, mercury sulfate, cadmium, Permanent Red 4R, Lithol Red, Pyrazolone Red, Watchung Red, calcium salt, Lake Red C, Lake Red D, Brillant Carmine 6B, Brillant Carmine 3B, Eoxine Lake, Rhodamine Lake B, or Alizarin Lake, blue pigments such as Miori Blue, Cobalt Blue, Alkali Blue Lake, Victoria Blue Lake, Phthalocyanine Blue, Non-metal Phthalocyanine Blue, partly chlorido Phthalocyanine Blue, Fast Sky Blue, and Indanthrene Blue BC; violet pigments such as Manganese Violet, Fast Violet B, or Methyl Violet Lake, green pigments such as chromium oxide, Chrome Green, Pigment Green B, Malachite Green Lake, and Finial Yellow Green G; white pigments such as Zinc White, titanium oxide, Anilomine White, zinc sulfide, and extender pigments such as beryllia powder, berillium carbonate, clay, silicon, white carbon, talc, and Alumina White. Of course, the granular base material is not limited to these substances. These substances can be used in a combination of two substances or more, as necessary. The shape of the base material can be selected, as necessary, dependent on its application and, for example, it is good to use a particle with a particle size of 0.1 μm to 1 mm.

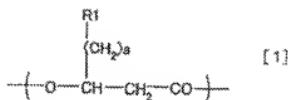
[0028] On the other hand, if the reaction is carried out by way of the fatty acid synthesis pathway, it can be considered that PHA is similarly synthesized by the PHA synthesizing enzyme using as a substrate (R)-3-hydroxyacyl CoA into which (R)-3-hydroxyacyl-ACP (ACP means an acyl carrier protein) produced in the pathway has been converted.

[0028] In addition, it is known that the above described PHB synthesizing enzyme and PHA synthesizing enzyme can be taken out from the cell to synthesize PHA in a cell-free system (*in vitro*), and specific examples thereof will be described below.

[0030] For example, in Proc. Natl. Acad. Sci. USA, 92, 6279-6283 (1995), it is reported that PHB comprising a 3-hydroxy-n-butyric acid unit has been successfully synthesized by making 3-hydroxybutyryl CoA act on a PHB synthesizing enzyme derived from *Alcaligenes eutrophus*. In addition, it is reported in Int. J. Biol. Macromol., 25, 55-60 (1999) that PHA comprising a 3-hydroxy-n-butyryl acid unit or a 3-hydroxy-n-valeric acid unit has been successfully synthesized by making 3-hydroxybutyryl CoA and 3-hydroxyvaleryl CoA act on the PHB synthesizing enzyme derived from *Alcaligenes eutrophus*. In addition, according to this report, when racemic 3-hydroxybutyryl CoA was made to act on the enzyme, PHB comprising only a 3-hydroxy-n-butyric acid unit of R-configuration was synthesized due to the stereoselectivity of the enzyme. Synthesis of PHB outside the cell using a PHB synthesizing enzyme derived from *Alcaligenes eutrophus* is also reported in Macromol. Rapid Commun., 21, 77-84 (2000). In addition, it is reported in FEMS Microbiol. Lett., 158, 319-324 (1998) that PHB comprising a 3-hydroxy-n-butyric acid unit has been successfully synthesized by making 3-hydroxybutyryl CoA act on a PHB synthesizing enzyme derived from *Chromatium vinosum*. It is reported in Appl. Microbiol. Biotechnol., 54, 37-43 (2000) that PHA comprising a 3-hydroxydecanoic acid unit has been synthesized by making 3-hydroxydecanoyl CoA act on a PHA synthesizing enzyme from *Pseudomonas aeruginosa*.

[0031] In this way, the PHA synthesizing enzyme is an enzyme catalyzing a final stage in the PHA synthesis reaction system in an organism, and any PHA known to be capable of being synthesized in the organism is synthesized under catalytic action by the enzyme. Therefore, by making 3-hydroxyacyl CoA corresponding to desired PHA act on the enzyme fixed on the medium in the present invention, pigment-coated capsular structure with any type of PHA known to be capable of being synthesized in the organism can be prepared.

[0032] As an example of PHA for use in the present invention, PHA containing at least monomer units expressed by the following formulae [1] to [10] can specifically be shown



35 (wherein the monomer unit is at least one selected from the group consisting of monomer units having any of the following combinations of R1 and a:

- 40 a monomer unit in which R1 represents a hydrogen atom (H), and a represents an integer number of 0 to 10;
a monomer unit in which R1 represents a halogen atom, and a represents an integer number of 1 to 10;
a monomer unit in which R1 represents a chromophoric group, and a represents an integer number of 1 to 10;
a monomer unit in which R1 represents a carboxyl group or a salt thereof, and a represents an integer number of 1 to 10, and
45 a monomer unit in which R1 represents,



45 and a represents an integer number of 1 to 7.)

containing structure can be obtained. Examples of such chromophoric groups may include nitroso, nitro, azo, diarylmethane, triarylmethane, xanthene, acridine, quinoline, methine, thiazole, indamine, indophenol, lactone, amideketone, hydroxylketone, stilbene, azine, oxazine, thiazin anthraquinone, phthalocyanine and indigo.

[0040] For PHA to be used in the present invention, random copolymers and block copolymers each including the above described plurality of monomer units can also be used, thus making it possible to control properties of PHA and provide a plurality of functions using the properties of respective monomer units and contained functional groups, to realize new functions using interaction between functional groups, and so on. In addition, it is also possible to synthesize a block copolymer of any order and composition on the surface of the base material by selecting an appropriate the amount and order in which 3-hydroxyacyl CoA as a substrate is added. In addition, as required, chemical modification and the like may also be made after or during synthesis of PHA.

[0041] It is also possible to change the composition of the monomer unit of PHA in the laminating direction of the polyhydroxylalkanoate-containing structure to the outside thereof by changing with time the composition such as type and concentration of 3-hydroxyacyl CoA as a substrate, for example. Thereby, for example, if it is necessary to form a coated structure with PHA having a low affinity for the base material, the base material is first covered with PHA having a high affinity for the base material, and the composition of the monomer unit of PHA having a high affinity for the base material is changed to the composition of the monomer unit of desired PHA in the laminated direction to form, for example, a multi-layer structure or gradient structure, thereby making it possible to form a PHA cover with its bonding to the base material enhanced.

[0042] In addition, chemical modification of the PHA can provide polyhydroxylalkanoate-containing structure various properties of which are improved. For example, the incorporation of a graft chain into a PHA can give polyhydroxylalkanoate-containing structure in which at least part of the base material has been coated with the PHA being given a variety of properties attributable to the graft chain. Further, crosslinking the PHA can provide polyhydroxylalkanoate-containing structure in which at least part of the base material has been coated with the PHA given a variety of physicochemical properties (for example, mechanical strength, resistance to chemicals and heat resistance). The term, "chemical modification" as used in the present invention indicates the meaning that the molecular structure of a polymer substance is altered by allowing an intramolecular or intermolecular chemical reaction of the polymer substance or a chemical reaction between the polymer substance and another chemical substance. The term, "crosslinking" indicates the meaning that a polymer substance is chemically or physicochemically bonded intramolecularly or intermolecularly to form a network structure. Furthermore, a crosslinking agent refers to a substance having a certain reactivity with the aforementioned polymer substance which is added to carry out the above crosslinking reaction.

[0043] Furthermore, PHA synthesized by a PHA synthesizing enzyme, which is used in the structure of the present invention, is generally an isotactic polymer constituted only by a R-configuration.

[0044] 3-hydroxyacyl CoA as a synthesis substrate for PHA can be synthesized for use by a method appropriately selected from an *in vitro* synthesis method using enzymes, an *in vivo* synthesis method using organisms such as microorganisms and plants, a chemical synthesis method, and the like. In particular, the enzyme synthesis method is a method that is generally used for synthesis of the substrate, and known enzyme synthesis methods include a method using the following reaction using commercially available acyl CoA synthetase (Acyl CoA Ligase, E.C.6.2.1.3) (Eur. J. Biochem., 250, 482-489 (1997), Appl. Microbiol. Biotechnol., 54, 37-43 (2000), etc.).

acyl CoA synthetase



For the synthesis process using enzymes and organisms, a batch type synthesis method may be used, or series production may be carried out using immobilized enzymes and immobilized cells.

<PHA synthesizing enzymes and microorganisms for producing the enzymes>

[0045] For the PHA synthesizing enzyme for use in the present invention, an enzyme produced by a microorganism appropriately selected from microorganisms capable of producing the enzyme, or a transformant with the gene of a PHA synthesizing enzyme introduced into the host may be used.

[0046] For microorganisms for producing PHA synthesizing enzymes, PHB or PHBV producing microorganisms may be used, and as these microorganisms, *Burkholderia cepacia* KK01, *Ralstonia eutropha* TB64, *Alcaligenes* sp. TL2 that have been isolated by the inventors may be used in addition to *Aeromonas* sp., *Alcaligenes* sp., *Chromatium* sp., *Comamonas* sp., *Methyllobacterium* sp., *Paracoccus* sp., *Pseudomonas* sp. and the like. Furthermore, KK01, TB64 and TL2 are deposited as FERM BP-4235, FERM BP-6933 and FERM BP-6913, respectively, in National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository.

[0047] Also, as microorganisms for producing PHA synthesizing enzymes, microorganisms producing mol-PHA and

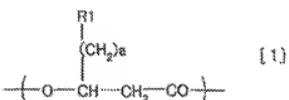
Claims

1. A method for manufacturing polyhydroxyalkanoate-containing structure, at least a part of a base material surface of the structure being coated with polyhydroxyalkanoate, the method comprising the steps of:

immobilizing a polyhydroxyalkanoate synthase on said base material surface,
synthesizing, on said base material surface, polyhydroxyalkanoate using a 3-hydroxyacyl coenzyme A to become the substrate of said synthase and said synthase and coating at least a part of said base material surface with the synthesized polyhydroxyalkanoate, wherein

said synthase contains an amino acid sequence capable of binding to said base material.

2. The manufacturing method according to claim 1, wherein the polyhydroxyalkanoate is comprised of at least one selected from the group consisting of monomer units expressed by Formulas [1] to [10], and each corresponding 3-hydroxyacyl coenzyme A is selected from the group consisting of 3-hydroxyacyl coenzymes A expressed by Formulas [12] to [21]:



(wherein symbol "a" represents an integer, and the combination of R₁ and "a" is selected from the group consisting of a combination of a hydrogen atom and any one integer selected from the group consisting of 0 to 10;

a combination of a halogen atom and any one integer selected from the group consisting of 1 to 10;
a combination of a chromiophoric group and any one integer selected from the group consisting of 1 to 10;
a combination of a carboxyl group or a salt thereof and any one integer selected from the group consisting of 1 to 10; and
a combination of



and any one integer selected from the group consisting of 1 to 7).